REMARKS

Claims 1-4, 6-9, 12, 13, 15-16, 26 and 34 are under examination. Claims 5, 10-11, 14, 17-25, 27-33, and 35-41 are cancelled. Applicants reserve the right to pursue the subject matter of the cancelled claims in a continuing application.

Claims 1-4, 6-9, 12, 13, 15-16, 26 and 34 have been amended to read "genetic skin diseases." Support may be found throughout the instant specification, for example, page 10, lns. 15-23, which lists examples of genetic skin diseases, and Figures 4-6 and Examples 1, 4, 5, and 6 which utilize skin or skin grafts for experimentation.

Claim 9 has been amended to reintroduce the phrase "junctional epidermolysis heredetaria bullosa, hemidesmosome epidermolysis bullosa hereditaria and ichthyosis congenita" in the light of the Examiner's withdrawal of the Restriction Requirement.

No new matter has been introduced by these amendments. Reconsideration and withdrawal of the rejections are respectfully requested in view of the above claim amendments and for the below reasons.

Response to Election/ Restrictions

Applicants acknowledge the Examiner's withdrawal of the restriction requirement, and has agreed that it would not be undue burdensome to examine the claims of all four groups together.

Response to 35 U.S.C. §112 Rejections

Claims 1-4, 6-9, 12, 13, 15-16, 26 and 34 were rejected under 35 U.S.C. §112, first paragraph as failing to comply with the enablement requirement. With regard to lack of

enablement requirement, the Examiner specifically proposed four issues in the outstanding Office Action.

(1) As a first issue, the Examiner contends that:

> ...the specification has provided no working example showing that disclosed remedy could be expressed at sustained levels by administering the naked DNA/liposome to any site by any method. Prior to instant invention, the art teaches a remedy for any genetic disorder involving plasmid DNA or liposome for correcting the deficiency of the gene was not predictable.
>
> (Office Action- p. 6)

To support this point, the Examiner cited Niidome, et al. (*Gene Ther.*, 9(24):1647-52, 2002) and Choate, et al. (*Hum. Gene Ther.*, 8(14):1659-65, 1997) and concluded as follows.

> It is evident from the cited art that the administration of plasmid DNA encoding any gene for correcting deficient gene for genetic disease by delivering naked DNA or liposome is not routine and remain unproven and unpredictable.
>
> (Office Action- pg. 7)

Applicants respectfully traverse the Examiner's assertion. Contrary to the Examiner's assertion, the specification of the instant invention shows that the disclosed remedy is expressed at sustained levels. Anti-Dsg3 IgG antibody production by transferring Dsg3 gene is demonstrated in Example 2 of the instant invention. Antibody production is shown to last for a long period of time (60 days) in Dsg3 knockout mice into which the Dsg3 gene was introduced by naked DNA injection method (Fig. 2). Since the presence of antigen (Dsg3) is reasonably

inferred from antibody (anti-Dsg3 IgG antibody) production, those skilled in the art understand that anti-Dsg3 IgG antibody was sustainably produced in the Dsg3 knockout mice as evidenced by Dsg3 gene being sustainably expressed in the Dsg3 knockout mice. Therefore, the instant specification shows that the disclosed remedy is expressed at sustained levels.

Even if the remedy of the instant invention was not sustainable, the problem could be solved with repeated administration of the remedy.

For these reasons, applicants believe that the Examiner's first issue regarding lack of enablement has been refuted.

(2) As a second issue, the Examiner pointed out at page 9 of the Office Action that:

> ...it is clear without any specific guidance on regulation of immune system and merely relying of CD40L antagonism is not enabling, because of the art, as shown above, does not disclose how B cell would correlate with T cell and how CD40 antagonism could results in sustained immune tolerance that is required for repeated dosing of naked DNA.

To support this point, the Examiner cited Miller et al. (*Transfus. Med. Hemother.*, 32:322-331, 2005) and Reipert et al. (*Throm. Hemost.*, 86:1345-1352, 2001). In response to the Examiner's assertion, applicants respectfully traverse in the following two points.

Firstly, the Examiner contends that B cell immune tolerance correlating with T cell immune tolerance is not clear, and relying only on antagonism of CD40L on the surface of T cell would not attain B cell immune tolerance. However, the instant specification at page 19, lines 13-20 states that:

> CD40-CD40L binding not only plays an important role in cellular immunity by promoting cytokine production and the like, but it is clarified to be quite important for

> proliferation of B cells, antibody production and the like. Thus, attempts have been made to suppress immune response in autoimmune diseases, organ transplantation and the like by using anti-CD40L monoclonal antibody inhibiting this binding.

To support this fact, Applicants have enclosed herewith Noelle, et al. (*Proc. Natl. Acad. Sci. USA*, 89:6550-6554, 1992) and Foy, et al. (*J. Exp. Med.*, 178:1567-1575, 1993) Noelle, et al. shows that a 39-kDa membrane protein (CD40L) is involved in T cell dependent B cell activation (see, abstract), and Foy et al. shows that humoral immunity is suppressed by blocking gp39 (CD40L)-CD40 interactions (see, abstract). As shown in these documents, the art acknowledges that the CD40L antagonist inhibits B cell proliferation and antibody production by blocking CD40L on the surface of T cells to bind with CD40 receptor on the surface of B cells, thereby attaining B cell immune tolerance. Therefore, anti-CD40L antibody is an appropriate immunosuppressive agent to attain both T cell immune tolerance and B cell immune tolerance, and the regulation of immune system using anti-CD40L antibody is enabling.

Secondly, the Examiner asserts that CD40L antagonism would not result in sustained immune tolerance and thus repeated dosing of naked DNA would not be enabling. However, Example 5 of the instant invention clearly shows that administration of anti-CD40L antibody into mice resulted in immune tolerance sustained for a long period of 42 days (See, Fig. 5). For this reason, those skilled in the art understand that sustained immune tolerance established with CD40L antagonism is enabling. Furthermore, even if CD40L antagonism should not attain sustained immune tolerance, it is possible to sustain immune tolerance with repeated administration of anti-CD40L antibody.

In the light of these arguments, applicants believe that the Examiner's second issue regarding lack of enablement has been refuted.

(3) As a third issue, the Examiner asserted as follows.

> Besides direct administration into skin, the specification provides no other specifics or showing that other routes of administration would result in expression in skin or other organs for the treatment of plurality of genetic disorder. (p. 10)

The Examiner contends that administration of naked DNA is not routine in the art, and since the specification does not provide any other method for administering naked DNA except "direct administration into skin," the claimed invention is allegedly not enabling. Radhakrishnan, et al. (WO 99/59638) cited by the Examiner regarding the novelty and obviousness rejections demonstrates that administration of naked DNA was routine at the time of filing the instant invention. The Examiner acknowledged this fact on page 17 of the Office Action, reciting that "[i]t would have only required routine experimentation to deliver gene as naked DNA/plasmid that was disclosed by Rdhakrishnan before filing this application." Applicants point to *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 231 USPQ 81 (Fed. Cir. 1986) for support that, "a patent need not teach, and preferably omits, what is well known in the art." Furthermore, as the Examiner is well aware:

> [e]nablement is a legal determination of whether a patent enables one skilled in the art to make and use the claimed invention. It is not precluded even if some experimentation is necessary, although the amount of experimentation needed must not be unduly extensive.
>
> (supra)

In view of this argument, applicants believe that the Examiner's third issue regarding lack of enablement has been refuted.

(4) As a fourth issue, the Examiner contends that:

> ...this disclosure does not provide sufficient guidance to an artisan to treat any genetic disorder. The instant claims read on treating any genetic disorder in any subject using the claimed remedy and method. However, it is unclear which conditions are treatable by the claimed remedy and method.
>
> (Office Action- p. 11)

To support this contention, the Examiner cited Passerson et al. (*Clinics in Dermatology*, 23(1):56-67, 2005). The Examiner contends that the remedy of the instant invention is not enabling because those skilled in the art would not be able to specify the diseases which are treatable by the claimed remedy. Applicants respectfully traverse the Examiner's contention. However, in order to expedite prosecution of the instant application, applicants have amended the claims such that they are presently directed to "genetic skin diseases." Those skilled in the art can thereby specify the diseases which are treatable by the claimed remedy. Thus, the instant invention is fully enabled.

In the light of the present claim amendments, applicants believe that the Examiner's fourth issue regarding lack of enablement has been rendered moot.

Applicants have addressed all of the issues regarding lack of enablement. Moreover, applicants assert that the instant specification describes the claimed invention with sufficient disclosure for one skilled in the art to make and use the invention as claimed. Reconsideration and withdrawal of the rejection under 35 U.S.C. §112, first paragraph are respectfully requested.

Response to 35 U.S.C. §102 Rejection

Claims 1-2, 6-8, 26 and 34 were rejected under 35 U.S.C. §102(b) as being anticipated by Radhakrishnan, et al. (WO 99/59638). Specifically, Radhakrishnan allegedly teaches the steps recited in the instant invention. Applicants respectfully traverse the Examiner's rejection.

As an initial matter, applicants have amended the claims of the instant invention to be directed to genetic skin diseases. Radhakrishnan, et al. do not describe a remedy for genetic skin diseases as claimed. Furthermore, applicants assert that Radhakrishnan does not disclose the claimed remedy and/or method of use.

remedy

The Examiner contends that Radhakrishnan, et al. disclose a vector to deliver nucleic acid molecules which include those encoding:

(1) immunomodulatory cofactors and

(2) protein required to replace a normal gene function capable of stabilizing or reversing inherited or non-inherited genetic disease such as CFTR, factor VIII and IX.

Radhakrishnan allegedly also discloses that the vector may direct the expression of at least two different recombinant or synthetic nucleic acid molecules such as immunomodulatory and therapeutic transgenes. Thus, the Examiner concludes that claims 1-2 and 6-7 are anticipated by Radhakrishnan, et al. Applicants respectfully traverse it in the following two points.

Firstly, Radhakrishnan, et al. describe "compositions and methods for the delivery of nucleic acid molecules" but not a "remedy" as claimed. Radhakrishnan, et al. essentially describes how to "facilitate" the uptake of nucleic acids. Specifically, the Radhakrishnan, et al.

describes "a recombinant or synthetic nucleic acid molecule in an aqueous solution comprising 0.5% to 5% (w/v) neutral or negatively charged polysaccharide, or in a lyophilized composition comprising a neutral or negatively charged polysaccharide" (pg. 2, lines 14-18). Radhakrisnan does not describe a recombinant or synthetic nucleic acid molecule alone, but includes the limitation "in an aqueous solution comprising 0.5% to 5% (w/v) neutral or negatively charged polysaccharide, or in a lyophilized composition comprising a neutral or negatively charged polysaccharide" (*supra*; emphasis added). In fact, what is substantially disclosed in Radhakrishnan, et al. is not a remedy to be used in gene therapy of genetic diseases as claimed, but is a nucleic acid uptake facilitator (Radhakrishnan- pg. 1, lns. 4-6).

This point is also supported by the Examples of Radhakrishnan, et al. If Radhakrishnan, et al. does disclose a "remedy," then data showing the effectiveness of the remedy should be demonstrated by comparing a) a group where the remedy is administered to b) a control group where the remedy is not administered. The Examiner points to in Figure 1 of Radhakrishnan, et al. as showing the method step of administering a naked DNA (Office Action- page 14). However, Figure 1 presents data showing the effectiveness of nucleic acid uptake facilitating by comparing groups to which luciferase DNA was administered with 'nucleic acid uptake facilitators' consisting of hyaluronic acid, maltodextrin and PEG to a control group to which luciferase DNA was administered with saline. Furthermore, it is clear from Figures 2, 3, 4, 7 and 8 of Radhakrishnan, et al. that their intention is to show the efficacy of a 'nucleic acid uptake facilitator,' not a 'remedy'. For these reasons, Radhakrishnan, et al. describes a nucleic acid uptake facilitator' and not a remedy to be used in gene therapy of genetic skin diseases as claimed in the instant invention.

Secondly, the Examples of Radhakrishnan, et al. do not support a remedy to be used in gene therapy of genetic skin diseases as demonstrated by the selected animal model. Jiang, et al. (*J. Invest. Dermatol.*, 124(3):xi-xiii, 2005), which the Examiner considers to be pertinent to the applicants' disclosure (Office Action- page 20), describe knockout mice or "transgenic animals, such as those generated by homozygous ablation of the gene in mice, often recapitulate the features of autosomal recessive human diseases, and can serve as suitable animal models for gene therapy" (p. xi, right column, lines 7-12). Radhakrishnan, et al. does not use knockout animals, let alone suitable animal models for gene therapy. If, however, Radhakrishnan, et al. had used EPO knockout mice and shown an increase of EPO levels, those mice might have been considered suitable animal models for gene therapy with EPO gene deficiency. However, Radhakrishnan, et al. did not disclose such knockout mice, but used animals which had no need for treatment and hence were clearly inappropriate for animal models for gene therapy.

In contrast, Dsg3 knockout mice of the instant invention are exactly what is described in Jiang as "suitable animal models for gene therapy." The instant invention discloses a remedy to be used in gene therapy of genetic diseases using these suitable animal models; whereas, Radhakrishnan does not.

In view of these arguments and amendments, applicants respectfully assert that a "remedy to be used in gene therapy of genetic skin diseases" of the instant invention is not anticipated by Radhakrishnan, et al. at all. Reconsideration and withdrawal of the §102 rejections are respectfully requested.

method

The Examiner further contends that Radhakrishnan, et al. anticipate a "method for treating genetic diseases" of the instant invention, because Figure 1 of Radhakrishnan, et al. allegedly disclose the same method step of administering a naked DNA as the instant invention (Office Action- page 14). As argued above, Radhakrishnan, et al. do not disclose any remedy to be used in gene therapy of genetic skin disease, and therefore also do not disclose any method for treating genetic skin diseases using such remedy as claimed. For the above reasons and claim amendments, applicants respectfully assert that a method for treating genetic skin diseases of the instant invention, as well as a remedy for use in gene therapy of genetic skin diseases, is not anticipated by Radhakrishnan, et al. Therefore, reconsideration and withdrawal of the 35 U.S.C. 102(b) rejections to claims 1-2, 6-8, 26 and 34 are respectfully requested.

Response to 35 U.S.C. §103 Rejections

Claims 1-4, 6-8, 12, 13, 15-16, 26, and 34 were rejected under 35 U.S.C. §103(a) as being unpatentable over Dwarki, et al. (WO 99/06562), Radhakrishnan, et al., and Takahama, et al. (EP 1142473).

Claims 1-4, 6-9, 12, 13, 15-16, 26 and 34 were also rejected under 35 U.S.C. §103(a) as being unpatentable over Dwarki, et al., Radhakrishnan, et al., Takahama, et al., and Chen, et al. (*JBC*, 275(32):24429-24435, 2000).

The Examiner contends that:

(1) Dwarki, et al. allegedly disclose a remedy comprising (i) an immunosuppressant comprising anti-CD40L antibody and (ii) a therapeutic gene, and a method

for treating genetic diseases by using the remedy, but differ from the instant invention by suggesting use of an adeno-associated virus (AAV) instead of naked DNA or liposome (Office Action- page 15).

(2) Radhakrishnan, et al. allegedly disclose a remedy comprising a vector to direct expression of proteins comprising therapeutic proteins and immunomodulatory cofactors, and a method for treating genetic diseases by using the remedy, but do not teach administering an antagonist that inhibits the interaction between a CD40 receptor and the ligand (Office Action- page 16).

(3) Takahama, et al. allegedly disclose a method for acquiring immunological tolerance to a foreign DNA that is useful for genetic disease therapy, but do not teach administering an antagonist that inhibits the interaction between a CD40 receptor and the ligand (Office Action- page 16).

(4) Chen, et al. allegedly disclose treating recessive dystrophic epidermolysis bullosa (RDEB) *in vitro*, but do not teach administering naked DNA for correcting the deficiency (Office Action- pages 18-19).

Relying on these assertions, the Examiner concluded that the claimed invention would have been obvious to one of ordinary skill in the art at the time of the invention, because it would have been obvious for those skilled in the art to modify the delivery method of the remedy disclosed by Dwarki, et al. to include the naked DNA/plasmid taught by Radhakrishnan, et al. The Examiner further combines Takahama, et al. and Chen, et al. for providing motivation for the modification because Takahama discloses a method for acquiring immunological tolerance and Chen for using the remedy to treat recessive genetic epidermolysis bullosa heredetaria dystrophica. Applicants disagree with and respectfully traverse the Examiner's contention.

Dwarki, et al. recite at page 3, second paragraph (emphasis added) as follows:

> Unfortunately, however, numerous experiments have demonstrated that after a single intramuscular injection of a rAAV vector, readministration of the rAAV vector does not lead to expression of the recombinant protein, even if it encodes a different recombinant protein. Gene delivery experiments performed with AAV vectors demonstrate that the intracellularly expressed AAV proteins elicit strong cell-mediated immune responses that eliminates the transduced cells. Even when a replication-defective AAV virion lacks all virally encoded genes, the virion is encapsulated in the AAV capsid proteins that are responsible for the entry into the cell and transport of the packaged DNA to the nucleus. Because AAV viruses are relatively ubiquitous and non-pathogenic, a majority of the population of animals and humans has been exposed to one or more of the seven serotypes of AAV and has developed an immune response thereto. This response would neutralize any attempted gene therapy that employed an AAV vector of the same serotype as the immunizing strain. Accordingly, it is an object of the present invention to develop a method for administering an AAV vector to a patient in need of somatic gene therapy, wherein protein expression would not be neutralized by the patients' immune system.

It is clear from the recitation above, especially the recitation "numerous experiments have demonstrated that after a single intramuscular injection of a rAAV vector, readministration of the rAAV vector does not lead to expression of the recombinant protein, even if it encodes a different recombinant protein," that Dwarki, et al. intend to suppress the immunity where an antigen is an AAV vector (or an AAV protein). In other words, Dwarki, et al. disclose that an immunosuppressant is needed because of the use of an AAV vector. Hence, one would conclude that it is not necessary to administer an immunosuppressant when an AAV vector is not used.

On the other hand, the instant invention, which uses naked DNA, administers an immunosuppressant to inhibit immunogenicity of proteins which are expressed as a result of gene therapy. The instant invention describes administering an immunosuppressant to suppress

production of antibodies against proteins which are expressed by genes delivered to patients in gene therapy.

There is no motivation to combine Dwarki, et al., which presupposes use of an AAV vector and Radhakrishnan, et al. related to 'nucleic acid uptake facilitators.' Even if naked DNA were used instead of an AAV vector in Dwarki, et al., those skilled in the art would not be motivated to use an immunosuppressant since Dwarki describes the use of an immunosuppressant because an AAV vector is used. Thus, the combination of Radhakrishnan, Takahama, and with or without Chen does not remedy the deficiencies of Dwarki.

As stated above, one skilled in the art would not be motivated to modify the delivery method of remedy disclosed by Dwarki, et al. to include naked DNA/plasmid taught by Radhakrishnan, et al., and therefore the claimed invention would not have been obvious to one of ordinary skill in the art at the time of the invention. In the light of the arguments and claim amendments above, applicants now believe that the Examiner's rejection based on 35 U.S.C. §103(a) has been refuted. Reconsideration and withdrawal of the rejection to claims are respectfully requested.

Applicants have addressed all the rejections the Examiner raised. Applicants respectfully request for withdrawal of those rejections and issue of notice of allowance for this application.

CONCLUSION

Based on the foregoing amendments and remarks, applicants respectfully request reconsideration and withdrawal of the rejection of claims and allowance of this application.

AUTHORIZATION

The Commissioner is hereby authorized to charge any additional fees which may be required for consideration of this Amendment to Deposit Account No. **13-4500**, Order No. 4439-4026.

In the event that an extension of time is required, or which may be required in addition to that requested in a petition for an extension of time, the Commissioner is requested to grant a petition for that extension of time which is required to make this response timely and is hereby authorized to charge any fee for such an extension of time or credit any overpayment for an extension of time to Deposit Account No. **13-4500**, Order No. 4439-4026.

Respectfully submitted,
MORGAN & FINNEGAN, L.L.P.

Dated: November 2, 2006 By: _____

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In Vivo CD40–gp39 Interactions Are Essential for Thymus-dependent Humoral Immunity. II. Prolonged Suppression of the Humoral Immune Response by an Antibody to the Ligand for CD40, gp39

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Summary

The ligand for CD40 has been recently identified as a 39-kd protein, gp39, expressed on the surface of activated $CD4^+$ T helper cells (Th). In vitro, soluble CD40 and anti-gp39 have been shown to block the ability of Th to activate B cells, suggesting that gp39–CD40 interactions are important to T cell–dependent B cell activation. Here it is shown that in vivo administration of anti-gp39 dramatically reduced both primary and secondary humoral immune responses to erythrocytes and soluble protein antigens without altering responses to the T-independent type II antigen, trinitrophenyl-Ficoll. Treatment of mice for 4 d with anti-gp39 inhibited the anti–sheep red blood cell (SRBC) response for at least 3 wk and inhibited the expression of all immunoglobulin isotypes in secondary responses to the protein antigen, keyhole limpet hemocyanin. To examine the direct effect of anti-gp39 on Th function, SRBC-immune Th cells from anti-gp39–treated mice were adoptively transferred and shown to be fully capable of providing help. These results suggest that anti-gp39 treatment does not cause Th deletion or anergy. Anti-gp39 may mediate its profound immunosuppressive effects on humoral immunity by blocking gp39–CD40 interactions. Moreover, these studies establish gp39-CD40 as an important receptor-ligand pair for the targeting of therapeutic antibodies to control thymus-dependent humoral responses.

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Thymus-dependent (TD)[1] humoral immunity requires the participation of $CD4^+$ Th, and constitutes an essential arm of host immune defense to disease. Studies by Mitchison, Claman, Benacerraf, and Raff suggested that interactions between Th and B cells in vivo are essential in the development of humoral immunity. Moreover, in vitro studies have demonstrated that T cell–dependent antibody responses require activation of T cells by dendritic cells followed by an interaction between activated Th cells and B cells (1, 2). The requirement for physical contact between Th and B cells in the humoral immune response cannot be replaced by lymphokines, as all combinations of lymphokines have proven ineffective at inducing resting B cell growth and differentiation. Therefore, a unique cell contact–dependent signal transpires as a consequence of the physical interactions between Th and B cells which induces B cell activation. This contact-dependent signal is believed to be transduced as a result of the binding of gp39 on activated Th to its receptor, CD40, on B cells (3).

CD40, a mitogenic receptor expressed on all mature B lymphocytes (4, 5), is a member of the nerve growth factor receptor (NGFR) family of receptors (6). The ligand for CD40, gp39, is a type II membrane protein that is homologous to TNF-α and -β (7, 8), other NGFR family ligands. Evidence that CD40 is an important mitogenic receptor on B cells is derived from studies that show highly efficient triggering of human B cells by anti-CD40 and cofactors such as anti-CD20, anti-Ig, and lymphokines (7–12). In the presence of these cofactors, anti-CD40 has been shown to initiate both B cell growth and differentiation. Similar to anti-CD40, gp39, expressed as a recombinant membrane or soluble protein, also activates B cells in the presence of costimulators (7, 13).

gp39 is transiently expressed on activated $CD4^+$ Th in vitro (14) and is induced in vivo on $CD4^+$ T cells as a result of antigen administration (15). The $CD4^+$ T cell population expressing gp39 in vivo has been localized in situ juxtaposed to B cells producing antibodies to the immunizing antigen (15). In vitro and in vivo data suggest that during

[1] *Abbreviations used in this paper:* Chi-L6, chimeric L6; HIg, hamster Ig; HIM, hyper IgM syndrome; TD, thymus dependent; TI, thymus independent.

the course of cognate Th–B interaction, transient expression of gp39 by $CD4^+$ T cells is the result of antigen presentation (16). Once expressed, gp39 binds to CD40 and reciprocally triggers B cell activation. The ability of a mAb specific for gp39, MR1, to block the capacity of gp39-bearing Th to activate B cells in vitro has implicated gp39 as an important molecule in T cell–dependent B cell activation (17).

Further evidence implicating gp39-CD40 involvement in humoral immune responses has recently been provided by several groups demonstrating that mutations in the gene encoding gp39 result in the inability of humans to respond to TD antigens (18–21). An immunodeficiency characterized by failure to mount TD humoral immune responses, hyper-IgM syndrome (HIM), results in the expression of a defective gp39 molecule that lacks CD40 binding capacity. Although the B lymphocytes from these patients are reported to be normal (18, 19, 21), mutations in the gp39 molecule interrupt B cell triggering through CD40 and subsequent B cell activation and Ig production.

The present study examines the ability of a mAb specific for gp39 to neutralize the function of gp39 in vivo. In vivo administration of anti-gp39 reduced primary as well as secondary antibody responses to exogenous TD antigens, but not the T-independent (TI)-type II antigen, TNP-Ficoll. Furthermore, short-term treatment with anti-gp39 produced prolonged suppression of humoral immune responses. Th cells from anti-gp39–treated mice were capable of providing help upon adoptive transfer, suggesting that anti-gp39 treatment did not result in deletion or anergy of responding Th in vivo. Evidence is presented supporting the hypothesis that anti-gp39 exerts its profound immunosuppressive effects by directly blocking gp39–CD40 interactions in vivo.

Materials and Methods

Animals

Female, 6–8-wk-old BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were used for the in vivo experiments presented in this study. Animals were maintained in the specific pathogen-free animal facility at Dartmouth Medical School.

T Helper Cell Clones (Th1)

D1.6, an $I\text{-}A^d$-restricted, rabbit Ig-specific Th1 clone (22) was obtained from Dr. David Parker (University of Massachusetts, Worcester, MA). In this paper, D1.6 will be referred to as Th1.

Reagents and Antibodies

MR1, hamster anti-murine gp39 mAb (17), was purified by DEAE HPLC from ascites fluid. Hamster Ig (HIg), used as a control antibody, was purified similarly from hamster serum (Accurate Chemical and Scientific Corp., Westbury, NY). RG7/7.6.HL, a mouse anti–rat κ chain (strongly cross-reactive with hamster κ chain) antibody, (RG7), (23) was conjugated with horseradish peroxidase or FITC and used as a secondary reagent to detect MR1 and HIg. Affinity-purified goat anti–mouse IgM, IgG1, IgG2a, IgG2b and IgG3 (Southern Biotechnology Associates, Birmingham, AL) were used as detection antibodies in the antigen-specific ELISAs, as well as in the total IgM and IgG1 ELISAs. EM95 (kindly provided by Dr. T. Waldschmidt, University of Iowa, Iowa City, IA) a monoclonal anti–murine IgE, was used as the detection antibody for the IgE anti-KLH ELISA. Chimeric L6 (Chi-L6), a humanized IgG1 specific for the tumor antigen L6 (24), was kindly provided by Bristol-Myers Squibb Pharmaceutical Research Institute. Anti-CD4, GK 1.5 (25) was prepared by HPLC purification of ascites fluid. SRBC were purchased from Colorado Serum Co. (Denver, CO). Sea plaque agarose for use in anti-SRBC plaque assay was obtained from FMC Corp. BioProducts (Rockland, MA). Baby rabbit complement was purchased from Cedarlane Laboratories Ltd. (Hornby, ON, Canada). KLH (from *Megathura crenulata*) was purchased from Calbiochem-Novabiochem (La Jolla, CA). CFA for immunizations was obtained from Sigma Chemical Co. (St. Louis, MO). TNP-SRBC, TNP-KLH, and TNP-BSA were prepared as previously described (26).

Immunizations for Generation of In Vivo Primary and Secondary Antibody Responses

Primary Immune Responses. For eliciting primary antibody responses to SRBC or TNP-SRBC, mice were immunized with 200 μl of 1% SRBC or TNP-SRBC suspension (i.v.). The IgM, anti-SRBC response was assayed 5 d after administration of antigen using a modification of the Jerne plaque assay (27). IgM anti-TNP responses were measured by ELISA on day 6. Primary responses to the heterologous Ig Chi-L6 were generated by intraperitoneal immunization of 100 μg Chi-L6 on alum per mouse. The serum IgM anti-Chi-L6 antibody response was measured after 7 d. Primary responses to TNP-Ficoll were generated by immunization with 25 μg of TNP-Ficoll, i.p. The IgM anti-TNP response was measured on day 6 by ELISA.

Secondary Immune Responses. For generation of secondary humoral responses to KLH, animals were immunized with KLH in CFA (50 μg, i.p.). Mice were subsequently challenged with 10 μg of soluble KLH (i.p.) 3 mo later. The anti-KLH antibody response was measured on day 7 from the serum of immune mice utilizing isotype-specific ELISAs. Secondary antibody responses to Chi-L6 were generated by challenging Chi-L6 immune mice with 10 μg soluble Chi-L6, i.p. The serum IgG1 anti-Chi-L6 antibody response was measured after 7 d.

Anti-gp39 Treatment

Sterile, HPLC-purified anti-gp39 (MR1) or HIg (as an antibody control) was administered intraperitoneally on days 0, 2, and 4 after immunization or challenge as indicated for each experiment.

Antigen-specific ELISAs

The antigen-specific IgM, IgG1, IgG2a, IgG2b, IgG3, and IgE antibody titers were determined using isotype-specific ELISAs. Briefly, antigen, (1 mg/ml of KLH, Chi-L6, TNP_{16}-BSA, or TNP_3-BSA in PBS) was absorbed onto flexible polyvinyl microtiter dishes, overnight at 4°C. Plates were washed and blocked with PBS-1% FCS-sodium-azide. Diluted serum samples were incubated for 2 h at 37°C. Samples were washed and the antigen-specific antibody titers determined with one of the following alkaline-phosphatase–conjugated detection antibodies: goat anti–mouse IgM, IgG1, IgG2a, IgG2b, or IgG3 (Southern Biotechnology Associates). The IgE-specific ELISA was detected using biotin-conjugated EM95 followed by alkaline-phosphatase avidin (Zymed, South San Francisco, CA). All ELISAs were developed by reaction of alkaline-phosphatase with phosphatase substrate (Sigma Chemical Co.). Plates were analyzed on an ELISA reader (model MR700; Dynatech

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Laboratories Inc., Chantilly, VA) at 410 nm. Units represent arbitrary values based on the titration curve of a standard immune serum. All experimental groups were titered from 1:100 to 1:100,000, and the titer ascertained based on multiple point analysis. The levels of anti-KLH, anti-Chi-L6, and anti-TNP antibodies in unchallenged controls were below detection.

Detection of Serum Anti-gp39

Quantitation of Intact Anti-gp39 in the Serum of Anti-gp39–treated Mice. Serum from mice receiving 750 μg anti-gp39 (250 μg on days 0, 2, and 4) was obtained on days 7, 14, and 21 after initiation of anti-gp39 treatment. The serum was run on a 7.5% SDS gel under nonreducing conditions, transferred to nitrocellulose, and blotted with HRPO-conjugated RG7. After chemiluminescent detection, areas of the blot corresponding to 150–165 kD were scanned and digitized using an Apple Scanner and the Image 4.1 software program (Apple Computer, Inc., Cupertino, CA).

Analysis for Biologically Active Anti-gp39 in the Serum of Treated Mice. Anti-CD3–activated Th1, which express gp39, were stained with dilutions of serum from mice receiving 750 μg anti-gp39 (250 μg on days 0, 2, and 4) to determine the amount of biologically active gp39 remaining in the serum. Titrations of serum containing anti-gp39 were incubated with activated Th1 cell clones for 30 min at 4°C, followed by washing and subsequent incubation with FITC-RG7 for 30 min at 4°C. A standard curve of mean fluorescence intensity vs anti-gp39 concentration was generated using purified anti-gp39. Samples were analyzed on a FACScan® (Becton Dickinson & Co., Mountain View, CA) and the percent anti-gp39 remaining in the serum was deduced based on the anti-gp39 standard curve. The level of anti-gp39 present in the serum at d7 was set at 100%.

Adoptive Transfer of Th Cells

Mice were immunized intravenously with 200 μl of 1% SRBC, and administered anti-gp39 or HIg (250 μg on days 0, 2, and 4). On day 7 the splenocytes from nonimmune or SRBC-immune mice were removed, erythrocyte depleted, washed, and transferred intravenously (50×10^6/mouse) into irradiated recipients (600 rad) with or without 50×10^6 spleen cells from TNP-KLH primed (TNP-KLH-CFA, 50 μg, i.p.) mice as a source of immune B cells. At the time of transfer, mice were immunized intravenously with 200 μl of 1% TNP-SRBC. Serum IgG1 anti-TNP titers were ascertained on day 6 after transfer.

Results

Anti-gp39 Inhibits the Generation of Primary Antibody Responses to Erythrocyte Antigens. The impaired TD immunity observed in patients with HIM, as well as the potent inhibitory effects of anti-gp39 and CD40-Ig on Th-dependent B cell activation in vitro, provided the basis for the study of the potential immunosuppressive effects of anti-gp39 on humoral-mediated immunity in vivo. To investigate the role of gp39–CD40 interactions in primary TD humoral immune responses, the effect of in vivo administration of anti-gp39 on the primary antibody response to SRBC was determined. Animals were immunized with SRBC and administered anti-gp39 mAb (or control HIg) over the course of 4 d. On day 5, the primary anti-SRBC antibody response of anti-gp39–treated, HIg-treated, and control mice was ascertained. The IgM anti-SRBC plaque-forming cell (PFC) response of mice that received a total of 1.5 mg of anti-gp39 (500 μg/mouse on days 0, 2, and 4) was reduced 99% when compared to the anti-SRBC PFC response from control or HIg-treated mice (Fig. 1 *A*). In addition, administration of as little as

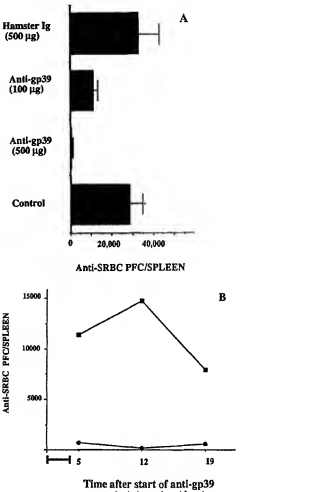

Figure 1. (*A*) Anti-gp39 inhibits the generation of primary anti-SRBC PFC. Mice (three per group) were administered 200 μl of 1.0% SRBC, i.v., on day 0. On days 0, 2, and 4 mice were given either 100 or 500 μg of purified MR1 (hamster anti-murine gp39, purified from ascites by DEAE HPLC) or 500 μg of purified hamster Ig, i.p. The control group consists of mice receiving the immunization, but no antibody treatment. Spleens were removed from the mice on day 5 and the number of direct (IgM) anti-SRBC PFC was determined by a modification of the Jerne plaque assay. The data is representative of three such experiments. (*B*) Prolonged immune suppression of primary anti-SRBC responses is induced by the administration of anti-gp39. Mice (three per group) were immunized with SRBC (200 μl of 1.0% SRBC, i.v.) and on day 0, 2, and 4, received 250 μg of anti-gp39 (●) or 250 μg hamster Ig (■), i.p. (*Black bar*) The time of antibody administration. The anti-SRBC PFC response was determined on day 5 after immunization. Additional mice were challenged with antigen (200 μl of 1.0% SRBC i.v.) 7 or 14 d after initial antigen immunization and anti-gp39 administration. The anti-SRBC PFC was then assayed 5 d later. The results are representative of three similar experiments.

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300 μg/mouse (100 μg/mouse on days 0, 2, and 4) of anti-gp39, reduced the anti-SRBC primary immune response by 66%. Results from these experiments demonstrate that anti-gp39 treatment ablates primary antibody responses in vivo.

The duration of the immunosuppressive effects of anti-gp39 on the primary humoral immune response to SRBC was subsequently examined. Mice immunized with SRBC were treated with anti-gp39 for 4 d and assayed at various later time points for the capacity to mount a primary anti-SRBC response. In this set of experiments, all animals were immunized with SRBC on day 0 and administered anti-gp39 or HIg on days 0, 2, and 4. The IgM anti-SRBC PFC response was measured for one group on day 5. Additional SRBC-immune groups were challenged with SRBC on day 7 or 14. 5 d after each antigenic challenge (days 12 and 19, respectively), the IgM anti-SRBC PFC response was measured. The results of one such experiment are depicted in Fig. 1 *B*. As in Fig. 1 *A*, the primary anti-SRBC responses were inhibited 80–90% 5 d after anti-gp39 administration was begun. In addition, the primary anti-SRBC responses 12 and 19 d after anti-gp39 treatment were also inhibited >90%. These results demonstrate that brief anti-gp39 treatment results in a prolonged inhibition of primary antibody responses.

Anti-gp39 Inhibits the Generation of Secondary Anti-KLH Antibody Responses. Experiments examining primary antibody responses suggest that gp39–CD40 interactions play a critical role in the initiation of primary humoral immunity. However, these experiments do not address whether gp39-dependent CD40 signaling is required for the generation of secondary antibody responses. Therefore, the effects of anti-gp39 administration on the secondary immune response to soluble challenge with KLH was determined in KLH-immune mice.

Using schedules of anti-gp39 administration that reduced the primary anti-SRBC PFC response, experiments were designed to evaluate the effects of anti-gp39 treatment on the secondary antibody responses. In these experiments, KLH-immune mice (immunized 3 mo before with CFA and KLH) were challenged with soluble KLH (10 μg/mouse/i.p.). On the day of antigen challenge (day 0), mice were also given 250 μg of anti-gp39 or HIg, followed by anti-gp39 or HIg on days 2 and 4. At days 7 (Fig. 2 *A*) and 14 (Fig. 2 *B*) after challenge with KLH, the mice were bled and the titers of

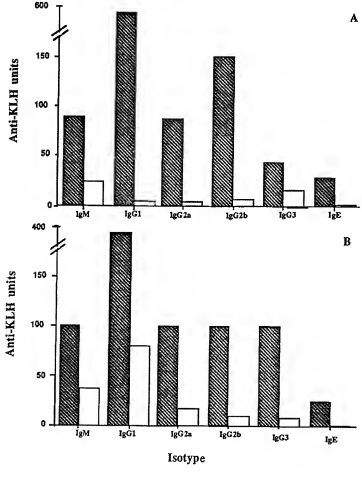

Figure 2. Anti-gp39 inhibits the generation of secondary anti-KLH antibody responses. Mice (three per group) were immunized with KLH in CFA (50 μg/mouse, i.p.). 3 mo after immunization, mice were given a soluble boost with 10 μg of KLH (i.p.). On days 0, 2, and 4, immune mice received 250 μg of anti-gp39, i.p. *(open bars)* or 250 μg HIg *(hatched bars)*. Serum from individual mice was collected on day 7 (*A*) or 14 (*B*) after antigenic challenge, pooled, and levels of anti-KLH antibodies were determined using isotype-specific ELISAs. Units represent arbitrary values based on the titration curve of a standard immune serum. All experimental groups were titered from 1:100 to 1:100,000 and the titer ascertained based on multiple point analysis. The levels of anti-KLH antibodies in unchallenged controls were below detection. The SE within each group were always <10%. These results are representative of three such experiments.

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IgM, IgG1, IgG2a, IgG2b, IgG3, and IgE anti-KLH antibodies were determined. The results demonstrate several points: (*a*) challenge with soluble KLH induced an enduring secondary immune response that persisted for up to 14 d; (*b*) the administration of anti-gp39 significantly reduced the secondary anti-KLH response of the isotypes measured when compared to the administration of equal quantities of HIg; and (*c*) the immunosuppressive effects of anti-gp39 appeared to be sustained for at least 14 d after the initiation of anti-gp39 treatment. Taken together, results from these experiments demonstrate that similar to primary humoral immune responses, the generation of secondary humoral immune responses were also blocked by anti-gp39.

Anti-gp39 Inhibits the Generation of Antibody Responses to Heterologous Ig. Experiments depicted in Fig. 1 demonstrate the immunosuppressive activity of anti-gp39 during a primary response to a strongly immunogenic particulate antigen, SRBC. The cellular nature of erythrocytes makes them unique in their capacity to elicit strong immune responses. Heterologous Ig molecules share this characteristic of being highly immunogenic, and therefore provide an additional model antigen system with which to examine the effects of anti-gp39 treatment on the generation of primary and secondary antibody responses. Animals were immunized with a heterologous Ig molecule, Chi-L6, a humanized mouse antitumor cell mAb, and treated with anti-gp39 or control HIg. After 7 d, sera was collected and assayed for the production of IgM anti-Chi-L6 antibodies. In addition, mice were challenged with Chi-L6 14 d after initial immunization and anti-gp39 treatment, and assayed for IgG1 anti-Chi-L6 antibody production on day 21. Fig. 3 depicts the results of one such experiment. The primary antibody response to Chi-L6 in mice treated with anti-gp39 is inhibited by >90% when compared to HIg-treated mice. Moreover, the secondary, IgG1 response to Chi-L6 is similarly inhibited. These results demonstrate that anti-gp39 treatment ablates primary and secondary antibody responses to a second type of TD antigen, heterologous Ig, as effectively as it suppresses responses to erythrocyte and soluble protein antigens.

Anti-gp39 Does Not Inhibit the Generation of Primary Antibody Responses to the TI-Type II Antigen, TNP-Ficoll. Although the previous experiments demonstrate that anti-gp39 effectively blocks the generation of primary and secondary antibody responses to TD antigens in vivo, it is unclear whether gp39–CD40 interactions play a role in the initiation of humoral responses to TI antigens. Data presented in the accompanying paper (15) demonstrate that immunization with the TI-type II antigen, TNP-Ficoll, results in gp39 expression by Th cells in vivo. To address whether gp39–CD40 interactions are necessary for the generation of antibody responses to this TI antigen, the affect of anti-gp39 treatment on mice immunized with TNP-Ficoll, was assessed. Mice immunized with TNP-Ficoll or TNP-SRBC were treated with anti-gp39 or HIg and the IgM anti-TNP antibody response determined after 6 d. Fig. 4 *A* demonstrates that animals immunized with the TD antigen TNP-SRBC elicit significant anti-TNP serum antibody responses. As predicted from the previously described experiments, anti-gp39 treatment dramatically inhibits the primary anti-TNP response generated in these mice. In contrast, mice immunized with TNP-Ficoll

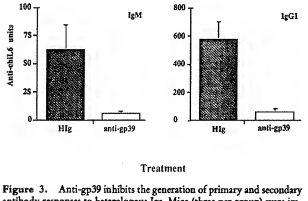

Figure 3. Anti-gp39 inhibits the generation of primary and secondary antibody responses to heterologous Igs. Mice (three per group) were immunized intraperitoneally with 100 μg Chi-L6 absorbed on alum. On days 0, 2, and 4, immune mice received 250 μg of anti-gp39, i.p. (*open bars*) or 250 μg HIg (*hatched bars*). Serum from individual mice was collected on day 7 after initial immunization (for *IgM*) or antigenic challenge (for *IgG1*). The levels of anti-Chi-L6 IgM and IgG1 antibodies were determined using antigen-specific ELISAs. Units represent arbitrary values based on the titration curve or a standard immune serum. All experimental groups were titered from 1:100 to 1:100,000 and the titer ascertained based on multiple point analysis. The levels of anti-Chi-L6 antibodies in unchallenged controls were below detection. The results are representative of two separate experiments.

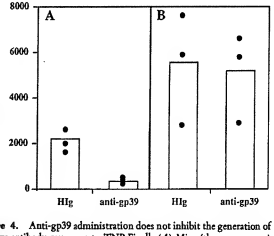

Figure 4. Anti-gp39 administration does not inhibit the generation of primary antibody responses to TNP-Ficoll. (*A*) Mice (three per group) were immunized with 200 μl 1% TNP-SRBC, i.v. On days 0, 2, and 4 mice received 250 μg anti-gp39 or HIg. On day 6, mice were bled and the IgM anti-TNP antibody titers determined by TNP_{16}-BSA ELISA. (*B*) Mice (three per group) were immunized with 25 μg TNP-Ficoll, i.v. On days 0, 2, and 4 mice received 250 μg anti-gp39 or HIg. On day 6 mice were bled and the IgM anti-TNP antibody titers determined by TNP_{16}-BSA ELISA. Units represent arbitrary values based on the titration curve or a standard immune serum. All experimental groups were titered from 1:100 to 1:100,000 and the titer ascertained based on multiple point analysis. The anti-TNP titer of nonimmune mice was 390 U. The results are representative of two separate experiments.

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mount a higher titered anti-TNP antibody response (Fig. 4 *B*); however, treatment with anti-gp39 does not inhibit the antibody response to TNP-Ficoll. Results from these experiments demonstrate that, unlike responses to TD antigens, anti-gp39 does not block the generation of humoral responses to TNP-Ficoll, suggesting that responses to TI antigens may be gp39 independent.

Anti-gp39 Administration Does Not Functionally Delete SRBC-specific Th. From the previous experiments, it is known that anti-gp39 interferes with the development of TD humoral immunity. However, the mechanism by which anti-gp39 treatment suppresses humoral responses is not clear. Immune suppression by anti-gp39 could be mediated by: (*a*) the negative signaling of gp39-bearing T cells causing Th anergy; (*b*) mAb-mediated cytotoxic deletion of anti-gp39 bearing $CD4^+$ T cells; and/or (*c*) the blocking of gp39 binding to CD40. A series of experiments were performed to gain insight into which of these mechanisms may be operative in the protracted immune suppression observed with anti-gp39 therapy. To explore the possibility that antigen-specific Th were deleted or anergized by anti-gp39 therapy, antigen-specific Th function from gp39-treated mice was measured by adoptive transfer. Briefly, mice were immunized with SRBC (to prime SRBC-specific Th) and administered anti-gp39 or HIg (250 μg/mouse on days 0, 2, and 4). After 7 d, spleen cells from unimmunized mice or SRBC-immune spleen cells from HIg- or anti-gp39–treated mice were adoptively transferred into recipient mice with TNP-immune spleen cells as a source of TNP-primed B cells. Mice were simultaneously challenged with TNP-SRBC, and the IgG1 anti-TNP titer ascertained on day 5. SRBC-primed Th cells are required to elicit a secondary anti-TNP response in the recipient mice as demonstrated by the fact that recipients that received spleen cells from nonimmune donors produced substantially lower IgG1 anti-TNP compared to those mice that received spleen cells from SRBC-primed animals (Fig. 5). More importantly, results of these experiments revealed that the SRBC helper activity from HIg- and anti-gp39–treated mice was similar, indicating that anti-gp39 treatment did not alter Th function or block the priming of Th. Moreover, antigen-responsive Th were not deleted or anergized as a result of anti-gp39 treatment, as they provided helper-effector function upon transfer.

In Vivo Clearance of Hamster Anti-gp39. Previous studies have established that anti-gp39 (MR1) blocks the binding of gp39 to CD40 (16) and thus support the hypothesis that the in vivo immunosuppressive effects of anti-gp39 are due to the blocking of gp39–CD40 interactions. If one assumes this hypothesis to be correct, the long-term immune suppression observed with anti-gp39 administration requires the persistence of anti-gp39 in the host. To determine if anti-gp39 could be detected for the period of time that immune suppression was evident, the in vivo clearance rate of anti-gp39 from serum was determined. Mice were given a regime of antibody (3× 250 μg anti-gp39) over the course of 4 d and assayed for the levels of serum anti-gp39 at 7, 14, and 21 d after the initiation of antibody administration. Western blot analysis for nonreduced MR1 (160 kD) indicated that intact, serum anti-gp39 could be detected for at least 21 d after the initiation of antibody treatment (Fig. 6 *A*). The serum concentration of anti-gp39 in animals at 21 d was ~5% (based on scanning densitometry), when compared to the signals derived from serum of animals analyzed 7 d after initiation of antibody therapy.

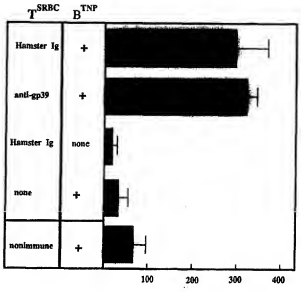

Figure 5. Anti-gp39 administration does not functionally delete SRBC-specific Th. Mice (three per group) were immunized with SRBC (200 μl of 1.0% SRBC, i.v.) and administered anti-gp39 or HIg (on days 0, 2, and 4; 250 μg/d). On day 7, the spleens from nonimmune or SRBC-immune mice were removed and transferred (i.v., 50 × 10^6/mouse) into irradiated recipients (600 rad) with/without 50 × 10^6 spleen cells from TNP-KLH primed (KLH/CFA 50 μg, i.v.) mice as a source of immune B cells. At the time of transfer, mice were also immunized with TNP-SRBC (200 μl of 1.0% TNP/SRBC). Serum IgG1 anti-TNP titers were ascertained on day 6 after transfer using a TNP_2-BSA ELISA. Units represent arbitrary values based on the titration curve of a standard immune serum. All experimental groups were titered from 1:100 to 1:100,000 and the titer ascertained based on multiple point analysis. The data are representative of two such experiments.

Although it was determined that intact anti-gp39 was present in serum, it was also important to ascertain that the anti-gp39 was biologically active. Therefore, sera from mice that received 3× 250 μg of anti-gp39 over the course of 4 d were used to stain gp39-bearing Th (Fig. 6 *B*). The level of serum anti-gp39 3 d after the last injection (7 d after initiation of antibody treatment) was set at 100%. 14 d after the initiation of antibody therapy, ~10–15% of the biologically active anti-gp39 mAb was detected in the serum. 21 d after initiation of therapy, 2–3% of anti-gp39 remained in the serum. Therefore, both the determination of intact gp39 by Western blotting and of biologically active anti-gp39 revealed that ~5% of the anti-gp39 was present 21 d after beginning anti-gp39 therapy. These results demonstrate the half-life of

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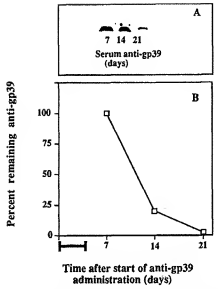

Figure 6. In vivo clearance of hamster anti-gp39. Mice were administered 3× 250 μg of anti-gp39 on days 0, 2, and 4. On days 7, 14, and 21, the amount of remaining anti-gp39 was determined as follows: (*A*) serum (1.5 μl) was electrophoresed under nonreducing conditions, transferred to nitrocellulose, and blotted with HRPO-conjugated RG7 (mouse anti-rat κ chain), followed by chemiluminescent detection. Areas of blot corresponding to 150–165 kD were scanned and digitized. (*B*) Titrations of serum were used to stain activated Th1 to determine the amount of biologically active anti-gp39 present in the serum. Activated Th1 were stained with titrations of serum followed by FITC-anti-rat κ chain (RG7). The percent anti-gp39 remaining in serum was deduced based on a standard curve of mean fluorescence intensity vs serum concentration, using day 7 as 100%. (*Black bar*) The time of antibody administration. The results are representative of two such experiments.

anti-gp39 to be approximately 12 d and offer evidence consistent with the hypothesis that prolonged suppression of humoral immune responses by anti-gp39 is due to persistent blocking of Th function.

Discussion

The present study demonstrates that in vivo administration of an anti-gp39 antibody which blocks gp39–CD40 interactions in vitro, results in profound inhibition of both primary and secondary humoral immune responses to TD antigens, but not TI-type II antigens. In addition, this study demonstrates that anti-gp39 treatment does not block the priming of antigen-primed Th cells. Therefore, the gp39–CD40 ligand-receptor pair can be used as a target for the therapeutic manipulation of the humoral immune response.

To gain insight into how anti-gp39 was exerting its immunosuppressive effect on humoral immunity, the direct effects of anti-gp39 on Th function were addressed. The data indicate that SRBC-immune Th from anti-gp39-treated mice were fully capable of providing help upon adoptive transfer, suggesting that anti-gp39 treatment did not cause Th deletion or anergy in vivo. These results led to the speculation that anti-gp39 mediates its immunosuppressive effects by blocking gp39 binding to CD40 and not by the inactivation of gp39-bearing Th. In support of this hypothesis, in vitro studies have established that anti-gp39 blocks the binding of CD40 to gp39 (17). Furthermore, biologically active anti-gp39 could be detected in serum for the period of time that immune suppression was apparent. Although only 5% of anti-gp39 was present in serum at a time when immune suppression was evident, it is possible that the local tissue concentrations of anti-gp39 in specific sites of secondary lymphoid organs is higher and clearance rates are slower than that of serum anti-gp39. Further insights are clearly needed to conclusively address the mechanism(s) of action of anti-gp39. Currently, studies are underway examining the effect of Fab and $F(ab')_2$ anti-gp39 on humoral immune responses so as to allow us to verify that anti-gp39–mediated inhibition is the result of gp39 blockade.

Treatment of mice with anti-gp39 inhibited the primary immune response to SRBC and heterologous Ig >90% for prolonged periods of time. Assuming that anti-gp39 is mediating the inhibition by blocking gp39 function, these data implicate gp39–CD40 interactions as essential in the development of primary immune responses to TD antigens. Immunohistochemical analysis establish that gp39 is induced as a consequence of immunization with TD antigens and may be of functional significance. The in situ studies of gp39 expression illustrate that the initial site of gp39–CD40 interactions during primary humoral immune responses is in the peripheral aspects of the periarteriolar lymphoid sheaths (PALS) and around the terminal arterioles (TA) of the spleen (15). It is at these sites that conjugates between gp39-expressing Th and antigen-specific B cells were found juxtaposed, suggesting that the outer PALS is a major site of T–B cell interactions during primary humoral immune responses. Therefore, the PALS may be the site at which anti-gp39 interacts with gp39-expressing Th cells to ultimately inhibit T-B interaction and subsequent Ig production. Immunohistochemical analysis of the distribution of anti-gp39 in anti-gp39–treated mice is underway to determine if this is the case.

Similar to primary responses, the secondary humoral immune response of mice primed to KLH in CFA was also shown to be inhibited by the administration of anti-gp39. Consistent with the reduction of anti-SRBC PFC by anti-gp39, reductions in serum antibodies titers to antigenic challenge were also observed. The serum titers of all anti-KLH Ig isotypes measured (IgM, IgG1, IgG2a, IgG2b, IgG3, and IgE) were reduced by the treatment of mice with anti-gp39. The effect of anti-gp39 administration was apparent for at least 14 d after secondary challenge with antigen, establishing a persistent immune suppression by anti-gp39. Anti-gp39–mediated immune suppression of secondary responses to KLH is not unique to KLH, since secondary immune responses to heterologous Ig and heterologous erythrocytes (data not shown) were also inhibited by anti-gp39 therapy. The anatomical distribution of gp39-expressing Th was identical to that observed upon primary immunization, however, the fre-

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quency of gp39-expressing Th in immune spleen was increased over that observed during primary immune responses. No gp39-expressing Th were found in the germinal centers or follicles of immune spleen (15). Thus, it appears that B cells are triggered to respond to activated Th cells in the PALS and TA of the spleen and later migrate to the follicles and germinal centers.

Although the in vivo studies demonstrate that anti-gp39 effectively blocks the generation of responses to TD antigens, it appears that gp39–CD40 interactions play little if any role in the initiation of humoral responses to antigens that have classically been characterized as T cell independent. Data presented in the accompanying paper demonstrate that gp39-expressing Th cells were found subsequent to immunization with both TD and one TI-type II antigen, TNP-Ficoll. However, based on our supposition that anti-gp39 is working via the blockade of gp39 function, the inability of anti-gp39 treatment to inhibit primary responses to TNP-Ficoll suggests that gp39–CD40 interactions are not required for the generation of humoral responses to TI-type II antigens. There are reports (15) that humoral immune responses to TNP-Ficoll are greatly augmented by Th. Therefore, it appears that the response to TNP-Ficoll requires Th but not gp39 function. A more comprehensive group of TI antigens are now under study to evaluate the gp39 dependence of these antigens for inducing humoral immune responses. Using anti-gp39, one should be able to refine the definition of TI and TD antigens as gp39 dependent or independent.

Recent studies on patients with HIM have provided genetic proof that gp39 is an essential component in TD humoral immunity (18–21). Patients with HIM are characterized by increased susceptibility to bacterial infections, associated with low levels of IgG, IgA, and IgE, a severe reduction in follicles and a complete lack of germinal centers (28). However, normal or increased levels of isohaemagglutinin, antityphoid, and Forrsman antibodies are typically observed in HIM patients. In these patients, vaccination often results in normal primary (IgM restricted) antibody responses, yet boosting rarely results in specific IgG responses (29). Given the results presented herein demonstrating that gp39 inactivation prohibits TD immune responses, the question emerges as to how patients with HIM, a genetic inactivation of gp39, mount primary IgM responses. First, many of the IgM responses observed in HIM patients may be due to TI type antigens eliciting restricted immunoglobulin isotype profiles. Our studies with TI antigens in mice indicate that the IgM responses to TI type II antigens are gp39 independent. Second, some antigens that we consider TD in the mouse, may act as TI antigens in humans. Third, it is not clear that all mutations in gp39 result in complete functional inactivation of the molecule and therefore some HIM patients may express partially functional TD responses. Indeed, it has been reported that activated T cells from at least one HIM patient weakly bound a soluble, recombinant form of CD40 (18). This observation supports the idea that some gp39 mutations may allow for the production of incomplete TD responses. Alternatively, one could suggest that gp39–CD40 interactions per se are not essential to the development of primary immune responses. Following this logic, one must then deduce that the immunosuppressive effects of anti-gp39 administration on the primary immune response are due to the deletion of activated, gp39-bearing Th. However, this is inconsistent with the data presented.

The focus of the present study was to demonstrate the potential use of anti-gp39 in the control of TD humoral immunity. Brief treatment regimes with the anti-gp39 resulted in prolonged suppression, an attractive attribute of this therapeutic antibody. Of special interest may be the capacity of anti-gp39 to prevent primary and secondary humoral responses to other heterologous, therapeutic antibodies such as Chi-L6. This would permit the exposure of patients to repeated administrations of heterologous therapeutic antibodies. Inhibitory effects on humoral immunity have been observed with other mAbs, i.e., anti-CD4 (27, 28). Although it is unclear how anti-CD4 mediates immune suppression, extensive deletion of $CD4^+$ T cells is correlated with suppressive efficacy (30), a phenomenon not observed with anti-gp39 therapy (data not shown). In addition to anti-CD4, it has been shown that the interference by CTLA-4 of CD28 triggering, a costimulatory molecule on Th cells, also suppresses TD antibody responses (31) and blocks xenogeneic graft rejection (32). Similar to anti-gp39 administration, CTLA-4 induced a state of prolonged immune suppression. Because anti-gp39 and CTLA-4 mediate their immunosuppressive effects at distinct stages of the humoral immune response, coadministration of these two immunosuppressive drugs may provide additive or synergistic immunosuppressive effects on immunity.

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Immunology

A 39-kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activation of B cells

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ABSTRACT **CD40 is a B-cell surface molecule that has been shown to induce B-cell growth upon ligation with monoclonal antibodies. This report shows that triggering via CD40 is essential for the activation of resting B cells by helper T cells (T_h). A soluble fusion protein of CD40 and human immunoglobulin, CD40-Ig, inhibited the induction of B-cell cycle entry, proliferation, and differentiation by activated T_h1 and T_h2. The ligand for CD40 was identified as a 39-kDa membrane protein that was selectively expressed on activated T_h. A monoclonal antibody specific for the 39-kDa protein inhibited CD40-Ig binding and also inhibited the activation of B cells by T_h. These data indicate that the 39-kDa membrane protein expressed on activated T_h is a binding protein for CD40 and functions to transduce the signal for T_h-dependent B-cell activation.**

Studies by Mitchison, Benacerraf, and Raff first suggested that physical interactions between helper T cells (T_h) and B cells were essential in the development of humoral immune responses. Later studies documented that T_h formed physical conjugates with class II major histocompatibility complex-compatible, antigen-presenting B cells (1) and that it was the B cells within these conjugates that responded to T_h (2). With the discovery that T_h-derived lymphokines exerted potent growth and differentiative effects on B cells, it was proposed that soluble factor(s) released in proximity by activated T_h mediated the activation of the interacting B cells. However, none of the molecularly cloned lymphokines, alone or in combination, manifested the ability to induce B-cell cycle entry. Unlike soluble factors, plasma membrane fractions from activated T_h induced B-cell cycle entry (3–5). Studies using purified plasma membranes from activated T_h (PM^{Act}) suggested that a protein expressed on the membrane of activated T_h cells was responsible for initiating humoral immunity (5, 6).

PM^{Act} have been used to investigate the nature of this effector function (4, 5). PM^{Act} from activated T_h, but not purified plasma membranes from resting T_h (PM^{Rest}), expressed an activity that induced B-cell cycle entry in an antigen-nonspecific, class II-unrestricted manner. Because of the lack of antigen specificity and class II restrictions, it was proposed that nonpolymorphic membrane protein(s) on activated T_h mediated the activation of interacting B cells. In addition, the activity expressed by PM^{Act} required 4–6 hr of activation and *de novo* RNA synthesis and was protein in nature (6). Here we show that activated T_h express a 39-kDa protein that binds CD40. Blocking the binding of this ligand to CD40 inhibited T_h-dependent B-cell activation. These data suggest that the binding of the 39-kDa protein on activated T_h to CD40 on B cells initiates thymus-dependent humoral immune responses.

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MATERIALS AND METHODS

Mice. Female DBA/2J mice (The Jackson Laboratory) were used for the preparation of filler cells to support the growth of T_h clones and in the preparation of resting B cells.

T_h Clones. D1.6, an I-A^d-restricted, rabbit Ig-specific T_h1 clone, and CDC35, an I-A^d-restricted, rabbit Ig-specific T_h2 clone, were obtained from David Parker (University of Massachusetts, Worcester). In this paper, D1.6 will be referred to as T_h1 and CDC35 as T_h2 (7).

Activation of T_h by Anti-CD3. T_h1 or T_h2 were cultured (8×10^6 per well) in cluster wells (six-well, Corning) coated with anti-CD3 (40 μg/4 ml of phosphate-buffered saline per well) for 16 hr, as described (5).

Preparation of T_h Plasma Membranes. Plasma membranes were prepared by discontinuous sucrose gradient sedimentation (5).

Preparation of Resting B Cells. Resting splenic B cells were prepared by sedimentation on discontinuous Percoll gradients (8). Cells isolated from the 70–75% (density, 1.087–1.097 g/ml) Percoll interface were typically >95% membrane Ig-positive, had a uniform, low degree of near forward light scatter, and were unresponsive to Con A.

Monoclonal Antibodies (mAbs). The following mAbs were purified by ion-exchange HPLC from ascites grown in mice that had been irradiated and bone marrow-reconstituted: anti-CD3, 145-2C11 (9); anti-$\alpha\beta$ T-cell antigen receptor (TCR), H57-597 (10); anti-CD4, GK1.5 (11); anti-ICAM-1, YN1/1.7.4 (12); anti-LFA-1, FD441.8 (13); and anti-rat/hamster Ig κ chain, RG-7 (14).

Preparation of the CD40 Recombinant Globulin (CD40–Ig). A plasmid containing a cDNA encoding the CD40 antigen (15) was digested with the restriction enzymes *Pst* I and *Sau*3A1. The *Pst* I–*Sau*3A1 fragment was subcloned into the same plasmid digested with *Pst* I and *Bam*HI. This allowed the preparation of a DNA fragment encoding a CD40 protein that was truncated upstream from the transmembrane domain. The fragment encoding the truncated CD40 was then subcloned into the Ig fusion plasmid (16) by using an *Mlu* I and *Bam*HI digest. The CD40–Ig fusion protein was produced by transient transfection in COS cells and purified on a protein A column (16).

Lymphokines. Recombinant mouse interleukin 4 (IL-4) was generously provided by C. Maliszewski and K. Grabstein (Immunex, Seattle). Recombinant mouse IL-5 was purchased from R&D Research (Sarrento, CA).

Induction of B-Cell RNA Synthesis by PM^{Act}. Resting B cells (3×10^6) were cultured in 50 μl of complete RPMI medium (RPMI 1640 plus 10% fetal bovine serum and 50 μM 2-mercaptoethanol) in A/2 microtiter wells (Costar). To these

Abbreviations: T_h, helper T cell(s); PM^{Act} and PM^{Rest}, purified plasma membranes from activated and resting T_h, respectively; TCR, T-cell antigen receptor; LPS, lipopolysaccharide; IL, interleukin; mAb, monoclonal antibody.

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wells, 0.5 μg of T_h1 or T_h2 membrane protein was added. Forty-two hours later 2.5 μCi (92.5 kBq) of [^{3}H]uridine (New England Nuclear) was added to each well. After 6 hr the cells were harvested, and the radioactivity was determined by liquid scintillation spectrometry. Results were expressed as cpm per culture (mean ± SD).

Induction of B-Cell Ig Secretion by PM^{Act} and Lymphokines. Resting B cells were cultured as described above. To each culture well, 0.5 μg of T_h1 membrane protein, IL-4 (10 ng/ml), and IL-5 (5 ng/ml) were added. On day 3 of culture, an additional 50 μl of complete RPMI was added. On day 6 of culture, supernatants from individual wells were harvested and quantitated for IgM and IgG1 (5).

Induction of B-Cell Proliferation by Activated T_h and IL-4. Resting B cells (4×10^4) were cultured in 50 μl of complete RPMI in A/2 microtiter wells (Costar). To each well, IL-4 (10 ng/ml) and 10^4 resting or activated irradiated (500 rads; 1 rad = 0.1 Gy) T_h1 were added. On day 3 of culture, cells were incubated with 1 μCi of [^{3}H]thymidine, and incorporation was determined as described (5).

Production of mAbs Specific for Membrane Proteins Induced on Activated T_h1. Hamsters were immunized intraperitoneally with $5–10 \times 10^6$ activated T_h1 (D1.6) at weekly intervals for 6 weeks. When the serum titer against murine T_h1 was >1:10,000, the hamster splenocytes and NS1 mouse myeloma cells were fused in the presence of polyethylene glycol. Supernatants from wells containing growing hybridomas were screened by flow cytometry for reactivity with resting and activated T_h1. One particular hybridoma, which produced a mAb that selectively recognized activated T_h, was further tested and subcloned to derive MR1. The MR1 mAb was produced in ascites and purified by ion-exchange HPLC.

Flow Cytofluorometric Analysis of Activation Molecules Expressed on T_h. Resting and activated T_h (16 hr with anti-CD3) were harvested and incubated at 10^5 cells per 50 μl with fusion protein for 20 min at 4°C, followed by fluorescein-conjugated goat anti-human IgG (25 μg/ml; Southern Biotechnology Associates, Birmingham, AL). Propidium iodide was added (2 μg/ml) to all samples. Flow cytofluorometric analysis was performed on a Becton Dickinson FACScan. After positive gating of cells by forward vs. side scatter, and by red negativity (for propidium iodide exclusion), the green fluorescence (logarithmic scale) of viable cells was ascertained. At least 5000 viable cells were analyzed for the determination of percent positive cells. Staining with MR1 employed fluorescein-conjugated RG7, a mouse anti-rat/hamster κ chain mAb.

Biosynthetic Labeling, Immunoprecipitation, SDS/PAGE, and Fluorography. T_h1 were rested or were activated with insolubilized anti-CD3 for 16 hr. Resting and activated T_h (20×10^6 per ml) were labeled with 1 mCi of [^{35}S]methionine/cysteine for 1 hr, washed twice in RPMI-1640 with 10% fetal bovine serum, and lysed in extraction buffer (17). Purified antibodies or fusion proteins (1–10 μg) were added to 500 μl of lysate (5×10^6 cell equivalents) at 4°C for 16 hr. At that time, the lysates were transferred to tubes containing 50 μl of packed protein A-Sepharose. The pelleted protein A-Sepharose was resuspended and tubes were incubated at 4°C for 1 hr with agitation. The samples were then washed three times with high-stringency wash buffer. The pelleted protein A-Sepharose was suspended in 30 μl of SDS sample buffer and run in a 10% polyacrylamide gel. After electrophoresis, the proteins were fixed in the gel and fluorography was performed.

RESULTS

Effect of mAbs on the Induction of B-Cell RNA Synthesis by PM^{Act}. To define the cell surface molecules that mediate the induction of B-cell cycle entry by PM^{Act}, mAbs to T_h membrane proteins were added to cultures of PM^{Act} and B cells. As previously published (5), PM^{Act} induced B-cell RNA synthesis 8-fold over that observed with PM^{Rest} (Fig. 1*A*). The addition of anti-LFA-1, anti-CD4, or anti-ICAM-1 alone, or in combination, did not inhibit induction of B-cell RNA synthesis by PM^{Act}.

CD40–Ig Inhibits T_h-Induced B-Cell Cycle Entry, Differentiation, and Proliferation. In the human system, it had been shown that anti-CD40 mAb induced B-cell proliferation (18), thereby implicating CD40 as an important triggering molecule for B cells. To determine whether CD40 was involved in the induction of B-cell RNA synthesis by PM^{Act}, a soluble fusion protein of the extracellular domains of human CD40 and the Fc domain of human IgG1 (CD40–Ig) was added to cultures of PM^{Act} and B cells. PM^{Act} derived from T_h1 and T_h2 were prepared and used to stimulate B-cell RNA synthesis. The addition of CD40–Ig to culture caused a dose-dependent inhibition of B-cell RNA synthesis that was induced by PM^{Act} from T_h1 and T_h2 (Fig. 1*B*). Half-maximal inhibition of B-cell RNA synthesis induced by PM^{Act} from T_h1 and T_h2 was achieved with about 5 μg of CD40–Ig per ml. A CD7E–Ig

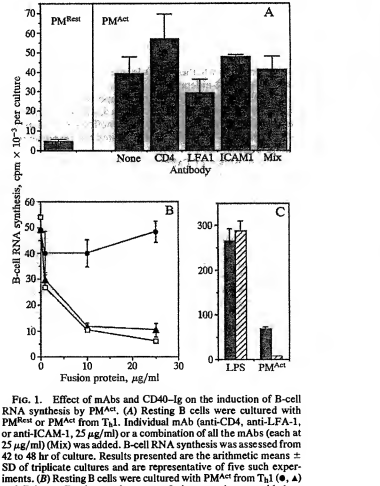

FIG. 1. Effect of mAbs and CD40–Ig on the induction of B-cell RNA synthesis by PM^{Act}. (*A*) Resting B cells were cultured with PM^{Rest} or PM^{Act} from T_h1. Individual mAb (anti-CD4, anti-LFA-1, or anti-ICAM-1, 25 μg/ml) or a combination of all the mAbs (each at 25 μg/ml) (Mix) was added. B-cell RNA synthesis was assessed from 42 to 48 hr of culture. Results presented are the arithmetic means ± SD of triplicate cultures and are representative of five such experiments. (*B*) Resting B cells were cultured with PM^{Act} from T_h1 (●, ▲) and T_h2 (○). To these cultures, no fusion protein was added or CD40–Ig (▲, □) or CD7E–Ig (●) was added at 1–25 μg/ml. B-cell RNA synthesis was assessed as in *A*. Results are the arithmetic means ± SD of triplicate cultures and are representative of three such experiments. (*C*) Resting B cells were cultured with LPS (50 μg/ml) or PM^{Act}. CD40–Ig (25 μg/ml; hatched bar) or CD7E–Ig (25 μg/ml; stippled bar) was added. B-cell RNA synthesis was assessed as *A*. Results are the arithmetic means ± SD of triplicate cultures and are representative of three such experiments.

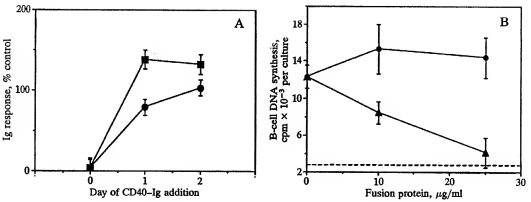

FIG. 2. CD40–Ig inhibits B-cell differentiation and proliferation. (*A*) Resting B cells were cultured with PM^{Act}, IL-4 (10 ng/ml), and IL-5 (5 ng/ml). Either at the initiation of culture, or on day 1, 2, or 3 after initiation of culture, CD40–Ig or CD7E–Ig (25 μg/ml) was added. On day 6 of culture, supernatants from individual wells were harvested and quantitated for IgM (●) and IgG1 (■) by anti-isotype-specific ELISA (5). In the presence of PM^{Act}, IL-4, and IL-5 (in the absence of added CD40–Ig), IgM and IgG1 were 4.6 μg/ml and 126 ng/ml, respectively. Cultures that received CD7E–Ig (25 μg/ml) on day 0 produced 2.4 μg/ml and 89 ng/ml of IgM and IgG1, respectively. The control of 100% is based on the response in the presence of CD7E–Ig. In the absence of IL-4 and IL-5, no IgM or IgG1 was detected. Results are representative of three such experiments. (*B*) T_h1 were rested or activated with anti-CD3 for 16 hr, irradiated, and cultured (10^4 per well) with resting B cells (4×10^4) in the presence of IL-4 (10 ng/ml). CD40–Ig (▲) or CD7E–Ig (●) was added at 0–25 μg/ml. From 66 to 72 hr of culture, cells were incubated with 1 μCi of [^{3}H]thymidine and then harvested. Broken line, response of B cells to resting T_h without the addition of fusion protein. Results are the arithmetic means ± SD of triplicate cultures and are representative of two such experiments.

fusion protein (19) was without effect even when used at 25 μg/ml.

To investigate whether CD40-Ig inhibited the activation of B cells by T-cell-independent activators, B cells were cultured in the presence of bacterial lipopolysaccharide (LPS; *Salmonella typhosa*) and CD40–Ig. On day 2, RNA synthesis was assessed (Fig. 1*C*). CD40–Ig was ineffective at inhibiting B-cell activation by LPS, yet it inhibited the response of B cells to PM^{Act}.

In the presence of PM^{Act}, IL-4, and IL-5, B cells polyclonally differentiated to produce Ig (4, 5). To evaluate the requirements for CD40 signaling in this process, CD40–Ig was added at the initiation of culture or on subsequent days of culture. The addition of CD40–Ig (Fig. 2*A*) at the initiation of culture inhibited >95% of the polyclonal IgM and IgG1 production compared with control levels in its absence. In contrast, the addition of CD40–Ig on day 1 or 2 of culture showed little if any inhibitory effect on IgM and IgG1 production. These data indicated that after 24 hr, signaling via CD40 was no longer essential for the differentiation of B cells to Ig secretion.

Data thus far implicated CD40 in the activation of B cells by PM^{Act}. Studies were performed to assure that CD40 was also involved in the activation of B cells by intact, viable, activated T_h. T_h1 were activated for 16 hr with insolubilized anti-CD3, harvested, and irradiated. The irradiated T_h1 were cultured with B cells in the presence of IL-4 and B-cell proliferation was determined on day 3 of culture. An exogenous source of IL-4 was required to achieve B-cell proliferation because T_h1 do not produce IL-4 (20). CD40–Ig inhibited the induction of B-cell proliferation by irradiated, activated T_h in a dose-dependent manner, similar to that observed with PM^{Act} (Fig. 2*B*). The negative control, CD7E–Ig, exerted no effect.

CD40–Ig Detects a Molecule Expressed on Activated T_h But Not on Resting T_h. To investigate whether activated T_h1 expressed a binding protein for CD40, resting and activated (16 hr) T_h1 were stained with CD40–Ig or CD7E–Ig, followed by fluorescein-conjugated anti-human IgG. Binding of CD40–Ig was assessed by flow cytometry (Fig. 3). Activated T_h1, but not resting T_h1, stained 56% positive with CD40–Ig, but not with the control CD7E–Ig. To identify the CD40–Ig-binding protein, T_h1 proteins were biosynthetically labeled with [^{35}S]methionine/cysteine and proteins were immunoprecipitated with CD40–Ig or CD7E–Ig. The immunoprecipitated proteins were resolved by SDS/PAGE and fluorography (Fig.

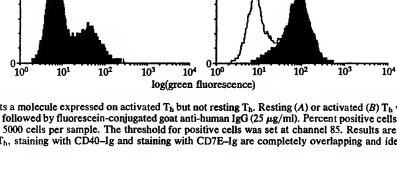

FIG. 3. CD40-Ig detects a molecule expressed on activated T_h but not resting T_h. Resting (*A*) or activated (*B*) T_h were incubated with fusion proteins for 20 min at 4°C, followed by fluorescein-conjugated goat anti-human IgG (25 μg/ml). Percent positive cells (ordinate) was determined by the analysis of at least 5000 cells per sample. The threshold for positive cells was set at channel 85. Results are representative of six such experiments. On resting T_h, staining with CD40–Ig and staining with CD7E–Ig are completely overlapping and identical in distribution.

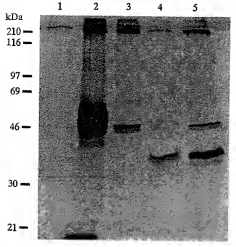

FIG. 4. CD40–Ig immunoprecipitated a 39-kDa protein from the lysate of activated T_h1. T_h1 were activated with insolubilized anti-CD3 for 16 hr. ^{35}S-labeled proteins from resting or activated T_h were immunoprecipitated without addition (lane 1), with purified anti-class I antibody (2.5 μg; lane 2), with CD7E–Ig fusion protein (10 μg; lane 3), or with CD40–Ig fusion protein (1 μg and 10 μg; lanes 4 and 5). Gel profile is representative of three such experiments.

4). A prominent band at 39 kDa was immunoprecipitated with 1 and 10 μg of CD40 per sample in a dose-dependent manner. As controls, anti-class I mAb immunoprecipitated bands at 55 kDa and a low molecular mass band, β_2-microglobulin. In the absence of mAb, no prominent bands were visible. A 39-kDa band was also immunoprecipitated from activated T_h that were vectorially labeled with ^{125}I, confirming that the 39-kDa protein was a membrane protein (data not shown). The immunoprecipitated 39-kDa band was identical in size when resolved by SDS/PAGE under nonreducing conditions, indicating that the CD40-binding protein was a single-chain molecule (data not shown).

A mAb Specific for the 39-kDa T_h Membrane Protein Inhibits the Induction of B-Cell RNA Synthesis by PM^{Act}. mAbs specific for antigens selectively expressed on activated vs. resting T_h were developed to identify T_h molecule(s) potentially responsible for T_h effector phase activity. One such mAb, MR1, recognized an antigen that was selectively expressed on activated T_h1. To investigate whether MR1 and CD40–Ig recognized the same molecule, flow cytometry and blocking studies were performed. CD40–Ig and MR1 stained approximately 56% and 61%, respectively, of activated T_h1, but not resting T_h1 (Table 1). MR1, but not another hamster

Table 1. mAb MR1 and CD40–Ig compete for the same protein on activated T_h

Staining mAb or fusion protein	Blocking mAb (conc., μg/ml)	% positive cells	
		Resting T_h	Activated T_h
MR1	—	5.7	61.8
CD40–Ig	—	4.9	56.5
	MR1 (5)	ND	49.5
	MR1 (10)	ND	30.3
	MR1 (25)	ND	20.7
	MR1 (50)	5.0	11.7
	Anti-$\alpha\beta$ TCR (50)	ND	63.0

Blocking studies were performed with flow cytometric analysis. Cells were incubated with hamster mAb MR1 (50 μg/ml) followed by fluorescein-conjugated anti-hamster κ chain or with CD40–Ig (50 μg/ml) in the presence of blocking mAb as indicated, followed by fluorescein-conjugated goat anti-human IgG1. Results are representative of three such experiments.

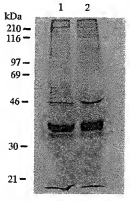

FIG. 5. MR1 and CD40–Ig recognize the same molecule in lysates of activated T_h. Proteins from [^{35}S]methionine-labeled activated T_h were immunoprecipitated with MR1 (10 μg per sample) (lane 2) or CD40–Ig (10 μg per sample) (lane 1) and resolved by PAGE and fluorography. Results are representative of two such experiments.

anti-T cell mAb, anti-$\alpha\beta$ TCR, blocked the staining of activated T_h1 with CD40–Ig, in a dose-dependent manner. These data suggested that CD40–Ig and MR1 recognized overlapping or identical epitopes on the 39-kDa T_h protein. To further investigate whether CD40–Ig and MR1 recognized the same molecule, the antigen that bound MR1 was identified by immunoprecipitation of radiolabeled proteins from T_h lysates. Both CD40–Ig and MR1 immunoprecipitated a 39-kDa protein (Fig. 5). Finally, immunoprecipitation of the 39-kDa protein with CD40–Ig removed the antigen recognized by MR1 from radiolabeled lysates of activated T_h (data not shown), supporting the idea that the MR1 antigen and the CD40 binding protein were identical.

Functional studies were performed with MR1 to address whether this mAb neutralized the activity expressed by PM^{Act}. PM^{Act} and B cells were cultured alone or in the presence of hamster mAbs or CD40–Ig. Two hamster mAbs, anti-$\alpha\beta$ TCR and anti-CD3, did not inhibit the activation of resting B cells by PM^{Act}. In contrast, MR1 or CD40–Ig inhibited B-cell activation (Fig. 6). MR1 did not inhibit LPS-induced B-cell RNA synthesis. B cells cultured with LPS incorporated 163,446 ± 3654 cpm per culture ([^3H]uridine) and in the presence of MR1 (25 μg/ml) incorporated 168,284 ± 8027 cpm per culture.

DISCUSSION

Blocking of prominent T_h surface molecules (LFA-1, CD4, ICAM-1, CD3, $\alpha\beta$ TCR) with mAbs did not impede the capacity of activated T_h to induce B-cell cycle entry. In

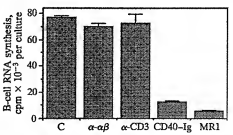

FIG. 6. A mAb specific for the induced 39-kDa T_h membrane protein inhibits the induction of B-cell RNA synthesis by PM^{Act}. Resting B cells and PM^{Act} were cultured without addition (control, C) or with anti-$\alpha\beta$ TCR (α-$\alpha\beta$), anti-CD3 (α-CD3), CD40–Ig, or MR1 (10 μg/ml). RNA synthesis was determined as described in Fig. 1. Results are the arithmetic means ± SD of triplicate cultures and are representative of three such experiments.

contrast, CD40–Ig or a mAb specific for the CD40-binding protein blocked T_h-dependent B-cell activation in a dose-dependent manner. Further, the CD40-binding protein was identified as a 39-kDa protein that was selectively expressed on the membranes of activated T_h but not resting T_h. Both CD40–Ig and a mAb specific for the 39-kDa CD40-binding protein blocked B-cell activation by PM^{Act}.

Although a number of membrane proteins have been implicated in T_h-dependent B-cell signaling, evidence presented herein dismisses the contribution of some molecules (LFA-1, CD4, CD3, $\alpha\beta$ TCR, ICAM-1) and implicates CD40 as the B-cell receptor for cognate signaling by T_h. Either CD40–Ig or a mAb specific for the CD40-binding protein inhibits T_h-dependent B-cell activation. CD40 was suspected to be an important ligand in T_h-dependent B-cell activation, since many functional responses of B cells to anti-CD40 mAbs and to activated T_h are similar. For example, anti-CD40 and PM^{Act} induce B-cell cycle entry (21–23). Further, anti-CD40 and activated T_h in the presence of IL-4 induce potent IgE production from resting B cells (21, 24–26). Finally, anti-CD40 and PM^{Act} induce homotypic B-cell aggregation (27). Therefore, both direct and indirect evidence implicates CD40 as the B-cell receptor for cognate help.

The ligand for CD40 is a 39-kDa protein that is expressed on activated, but not resting T_h. The 39-kDa protein is a single-chain molecule, since electrophoretic migration was not influenced by reducing agents (data not shown). Based on the functional studies presented in this study and preliminary biochemical studies (data not shown), activated T_h1 and T_h2 express the 39-kDa CD40-binding protein. This is consistent with the functional studies showing that both T_h1 and T_h2 induce B-cell cycle entry. To further characterize the 39-kDa protein, cDNA encoding CD proteins in the size range of 39 kDa (CD53 and CD27) were transiently transfected into COS cells and the cells were tested for CD40–Ig binding. None of the transfected COS cells expressed proteins that bound CD40–Ig. It is therefore suspected that the 39-kDa protein is not one of these CD proteins.

The biochemical basis for signal transduction between T_h and B cells has been elusive. The identification of CD40 as the signal-transducing molecule for T-cell help focuses attention on specific biochemical pathways known to be coupled to the CD40 molecule. CD40 is a member of the nerve growth factor receptor family by virtue of the presence of four cysteine-rich motifs in its extracellular region. Signaling through CD40 by mAb has been shown (28) to involve the activation of tyrosine kinases resulting in the increased production of inositol trisphosphate and the activation of at least four distinct serine/threonine kinases. Based on information obtained from signaling through other members of the nerve growth factor receptor family, it is thought that interaction between activated T_h and B cells results in many of the same biochemical processes.

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